Isolation & Characterization of Organic Molecules from Mississippian-age Crinoids

Honors Research Thesis

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by

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Abstract

In examples of exceptional preservation, fossil crinoids (phylum Echinodermata) from the same locality display species-specific color preservation. These colors are produced by light absorbing chromophores in organic molecules and have been recognized as polyaromatic hydrocarbons. Ultraviolet visible (UV-Vis) light spectroscopy and Orbitrap electrospray ionization mass spectrometry (ESI-MS) were used in this study to further elucidate the identity of these organic molecules, while also assessing the feasibility of utilizing these molecules as biomarkers to track phylogeny. Organic molecules extracted from fossil material were compared to extracts from modern echinoderm analogues in order to determine the most plausible identities. Results indicated that the organic molecules preserved in Paleozoic crinoids are distinct from those identified in Mesozoic crinoids. These differences may be attributed to biological differences between the different crinoid taxa, but most likely are associated with the different geologic settings that the crinoids were deposited in.

Acknowledgements

I must first recognize my advisors, Drs. Bill Ausich, Yo Chin, and Anne Carey, for without them I could not have done this project. Dr. Ausich has taught me so much in this last year and even managed to give me a true appreciation for echinoderms. Dr. Chin has been my advisor since my freshman year and has helped me progress not only in analytical chemistry, but independent research in general. Dr. Carey has also been an advisor of mine since my freshman year and has been significantly influential in my development as geologist. Dr. Carey has helped me with everything from scheduling classes to networking with employers. I have been extremely lucky to have such an amazing group of people to guide me through my undergraduate career.

Next I would like to thank all of those who helped me with my extractions and analyses. Dr. Chin's lab group really helped me get started with testing the simple extraction method and was extremely helpful with employing various analytical techniques. I have Christina O'Malley to thank for her advice on the use of Soxhlet extraction. Without Dr. O'Malley's help I don't know what results I would be presenting right now. I would also like to thank the CCIC for letting me use their mass spectrometer. I also really appreciated talking with Dr. Ausich and his lab group about echinoderms in group meetings. Those discussions were great in helping me understand a broad range of echinoderm related topics.

And another thanks to the Friends of Orton Hall whom helped to fund this project. Thank you to all of my advisors for all of the reference letters and support given to help gain additional funding for this project. In all honesty, I really should thank the whole Earth Sciences community for making OSU such a wonderful atmosphere. And finally, I would like to thank my friends and family from home that helped me reach this point.

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Introduction & Background

In examples of exceptional preservation, fossil crinoids (phylum Echinodermata) display species-specific color preservation (Figure 1). In 1931, Lowell R. Laudon stated that a part of the original color of these crinoids is still preserved in the specimens of the Hampton Formation in Le Grand, Iowa. However, this feature is now attributed to the preservation of biomarkers within the calcite mesodermal skeleton of the once living crinoids. These chemical traces of the molecules from Lower Mississippian crinoid fossils are the oldest known biomarkers extracted directly from fossil remains to date (O'Malley et al., 2013).

Figure 1: Crinoids from the Maynes Creek Formation of Le Grand, Iowa displaying species-specific coloration on single bedding plane. The lightest color crinoid is *Elegantocrinus symmetricus* and the two darker species are *Strimplecrinus inornatus* and *Cribancrinus watersianus* (O'Malley et al., 2013).

Commonly, coloration in fossils is thought to be the result of diagenesis and may be inorganic in nature, e.g. mineral oxides, yet that does not seem to be the case in the fossil

assemblages of Le Grand, Iowa; Crawfordsville, Indiana; and Monroe Reservoir, Indiana. Crinoids from these localities display species-specific coloration on a single bedding plane. If the coloration of the crinoids was a diagenetic affect, it would be expected that all the crinoids would be influenced in the same manner and, thus, would all display the same coloration. For this reason, these coloration patterns have been attributed to the preservation of organic molecules produced by the crinoids while living. This remarkable occurrence of species-specific coloration offers potential for a new approach to phylogeny and will undoubtedly shed light on the biochemistry of ancient echinoderms.

Research by many others has provided evidence of species-specific pigmentation in Triassic, Jurassic, and Mississippian fossil crinoids (Blumer, 1951; Blumer, 1960; Blumer, 1962; Thomas & Blumer, 1964; Blumer, 1965; O'Malley et al., 2005; O'Malley et al., 2006; Wolkenstein et al., 2006; O'Malley et al., 2009; and O'Malley et al., 2013). In the past, techniques such as ultraviolet-visibility light spectroscopy (UV-Vis), fluorescence spectroscopy, and mass spectrometry (MS) have been employed as a means of identifying the organic molecules preserved in the fossilized remains of crinoids. Yet, with each study new questions arose, and new analytical techniques are developed offering an opportunity to identify the molecules with greater accuracy. Through this study of Mississippian crinoid fossils, I will corroborate and extend the work of O'Malley et al. (2013) by 1) outlining a reproducible method for isolating organic molecules from fossil remains, 2) comparing modern and fossil pigments isolated from echinoderms, and 3) considering whether biomarkers can serve as a feasible means of reconstructing crinoid ancestry. The following information will serve as the necessary background in order to develop an understanding of how organic molecules preserve and what importance they may yet hold as fossilized remains.

Crinoid Physiology & Phylogeny

All echinoderms form tests (skeletons) composed of high-Mg calcite ossicles (plates) with a stereomic structure. Each ossicle is a single calcite crystal in optical continuity. In crinoids, these ossicles are articulated by soft tissue and ligaments to form the holdfast (root), the column (stem), and the crown (calyx and arms) (Hess et al., 1999; Figure 2). The stereom of a crinoid is a porous structure that is infilled with mesodermal tissue (stroma) while alive. The stroma accounts for approximately half of the volume of the echinoderm skeleton (Savarese et al., 1996). During early diagenesis, the mesodermal tissue is occluded with syntaxial calcite cement. Subsequently, the high-Mg calcite test is altered to geochemically stable low-Mg calcite (Brand, 1990). This calcite cement has been described as a crystal casket that protects the organic molecules from being destroyed by further diagenesis (Dickson, 2001).

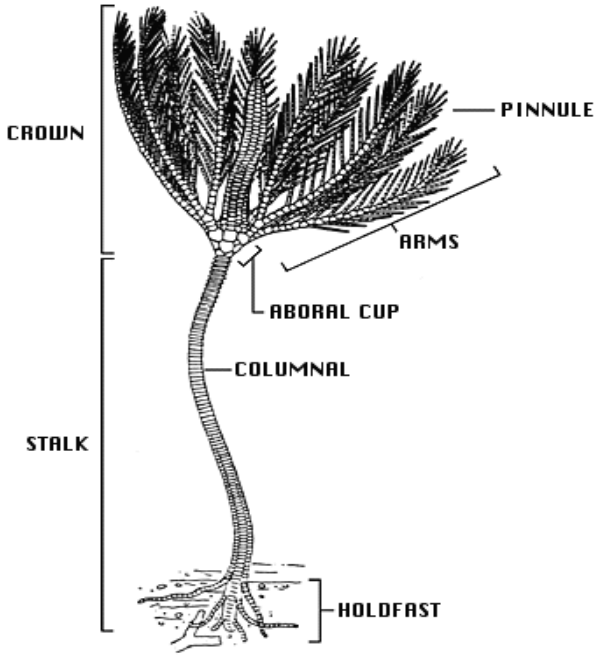


Figure 2: General morphology of a stalked crinoid (modified from Bather, 1900, copyright © 1998 William Ausich).

Crinoid ancestry is interpreted based on morphological characters such as holdfast attachment style, stem articulation, and calyx morphology. Ausich and Kammer discussed the progression of tegmen (oral plates) geometries as a means of understanding the evolutionary history of crinoid clades (Ausich et al., 2012; Kammer et al., 2012). Another approach to phylogeny to be considered has been proposed by O'Malley et al. (2013), in which biomarkers are used to determine crinoid ancestry.

Crinoids are separated into five subclasses: Camerata (Wachsmuth & Springer, 1885), Disparida (Moore & Laudon, 1943), Cladida (Moore & Laudon, 1943), Flexibilia, and Articulata (Ausich, 1998). Formerly, three subclasses were recognized: Camerata, Disparida, and Cladida (Simms & Sevastopulo, 1993). In yet another scheme by Moore & Teichert (1978), the cladids and the disparids were considered orders within the subclass Inadunata, and the subclass Echmatocrinea was recognized. The samples used in this study are from the camerates and the primitive cladids. In the modern classification scheme (Ausich, 1998), the camerate crinoids can be separated into two orders: Monobathrida and the Diplobathrida (Moore & Laudon, 1943). Crinoids of the genus *Elegantocrinus* (formerly *Platycrinites*; Miller, 1821) and *Strimplecrinus* are among the monobathrids and crinoids of the genus *Gilbertsocrinus* (Phillips, 1836) are among the diplobathrids. This study analyzes two species within the primitive cladids of the order Cyathocrinitida, which are crinoids of the genus *Cyathocrinites* and *Barycrinus* (Wachsmuth & Springer, 1868; Table 1).

Table 1: Phylogeny of the seven species of crinoids from this study.

Kingdom	Phylum	Class	Subclass	Order	Genus	Species
Animalia	Echinodermata	Crinoidea	Cladida	Cyathocrinitida	*Cyathocrinites*	*iowensis*
					Barycrinus	*rhombiferous*
			Camerata	Monobathrida	*Elegantocrinus*	*hemisphericus*
					Elegantocrinus	*symmetricus*
					Strimplecrinus	*inornatus*
				Diplobathrida	*Gilbertsocrinus*	*tuberosus*

Table 2: Species list with their associated localities, formations, and relative age.

Species	Locality	Formation	Age
G. tuberosus	Crawfordsville, IN	Edwardsville Fm.	early Visean (~340 Ma)
B. rhombiferous	Crawfordsville, IN	Edwardsville Fm.	early Visean (~340 Ma)
C. iownesis	Monroe County, IN (Boy Scout Camp)	Edwardsville Fm.	early Visean (~340 Ma)
E. hemisphaericus	Monroe County, IN (Boy Scout Camp)	Edwardsville Fm.	early Visean (~340 Ma)
E. symmetricus	Le Grand, IA	Maynes Creek Fm.	early Tournaisian (~355 Ma)
S. inornatus	Le Grand, IA	Maynes Creek Fm.	early Tournaisian (~355 Ma)

Geologic History

Fossil specimens were analyzed from three localities: Le Grand, Iowa; Crawfordsville, Indiana; and Monroe Reservoir, Indiana (Table 2). These localities were selected for their remarkable preservation. It is this characteristic exceptional preservation of a diverse fauna from these localities that earns them the title of *Lagerstätte*.

Forty species of crinoids have been identified from Le Grand, Iowa, two of which are analyzed in this study. The two species, in this study, from Le Grand, Iowa (*Strimplecrinus inornatus* and *Elegantocrinus symmetricus*) were all taken from a single bedding plane that lies in the Maynes Creek Formation of the Kinderhookian series, which is Tournaisian 2 in age (Anderson, 1969). The Maynes Creek Formation has abundant large slabs containing numerous species of crinoids that were deposited during a single episodic event in the Wassonville cycle (Gahn & Baumiller, 2004). Crinoids occur in the center of lenticular beds composed of relatively soft limestone (Laudon, 1931; Laudon & Beane, 1937; Hess, 1999). These lenticular deposits consisted of intraclastic crinoidal grainstone overlain by thin-bedded, dolomitic mudstone (Gahn & Baumiller, 2004). The crinoids have been interpreted to have lived in a nearshore carbonate shelf, in a marine surge channel that traversed a restricted tidal flat and oolitc shoals and skeletal grainstones (Gahn & Baumiller, 2002; Witzke & Bunker; 2002).

Fossil crinoids from Crawfordsville, Indiana and Monroe County, Indiana come from the Edwardsville Formation of the Borden Group, which is late Osagean in age (early Visean). The Borden Group was deposited as a low-relief delta supplied by sediment pulses eroding from the ancestral Appalachian Mountains. Quality of preservation varies throughout Indiana as a consequence of varying rates of sedimentation; yet at some localities, such as Crawfordsville, many crinoids are fully intact (crown, stem, and root) buried where they had once lived (Ausich

et al., 1979; Hess et al., 1999). There have been more than 63 crinoid species assigned to 42 genera that are known from Crawfordsville (Hess et al., 1999).

Samples in this study from Monroe County, Indiana come from a locality known as Boy Scout Camp within the Monroe Reservoir. The Borden delta platform at Monroe Reservoir was deeper than normal wave base, yet within the photic zone as proven by the presence of algae and within storm wave base (Ausich & Lane, 1980). The Borden delta was separated into five lithofacies along the Monroe Reservoir: subaqueous channel sandstanes, sheet sandstones, interchannel siltstones, interchannel mudstones, and skeletal carbonate banks (Ausich & Lane, 1980; Ausich, 1983). Each of these facies supported a unique faunal assemblage. Boy Scout Camp is within the interchannel mudstone facies, which supports the most diverse epifaunal suspension-feeding community at Monroe Reservoir (Ausich, 1983).

The Role of Pigmentation

Pigments are chemical substances that produce color by absorbing light. Chromophores are the reactive region of these pigments that produce color by exciting electrons from the ground state into an excited state through the absorption of ultraviolet or visible radiation. Modern taxa utilize pigmentation for numerous purposes such as camouflage, photosynthesis, mating, mimicry, etc. Animals such as the flounder or the chameleon are capable of changing the complexion of their skin in order to mimic the coloration of the surrounding environment. Many birds experience sexual dimorphism, in which the male and female have different coloration patterns. The males are often brilliantly colorful, and they use this feature to attract the eye of their female counterparts (i.e. cardinals). Chlorophyll is a green pigment, present in plants, algae, and cyanobacteria, that is utilized to absorb energy from light during photosynthesis. Color

serves as a major factor in the lives of just about everything, which is why developing an understanding of pigmentation throughout Earth history can reveal untold information regarding paleoecology.

Work on reconstructing pigmentation of ancient taxa is still in its infancy, but the practice is becoming increasingly popular. Vinther et al. (2008) reported the first evidence of preservation of a color-producing nanostructure, attributed to melanin, in a fossil feather. Melanin is an organic pigment derived from melanosomes that is ubiquitously found in bacteria, fungi, plants, and animals. Others have used nanostructures preserved in fossils to reconstruct the coloration patterns of extinct organisms. From the wavelengths reflected from layers of individual scales from fossilized moths (order Lepidoptera), it has been determined that a blue (hypsochromic) shift in wavelength occurs during the fossilization process (McNamara et al., 2011). This exemplifies the fact that the observable colors in fossils provide little to no indication of the living coloration in the organisms due to taphonomic alteration, yet the molecules still provide information regarding the biochemistry throughout Earth history. The next section will discuss our current understanding of organic molecule occurrence and preservation in echinoderms, from both extinct and extant taxa.

Biomarkers in Echinoderms

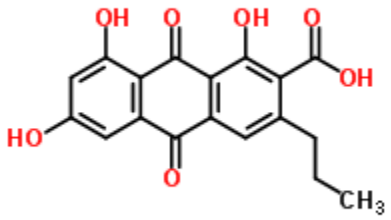
Studies on modern echinoderms have documented the occurrence of a variety of quinone pigments to be a characteristic feature of the phylum Echinodermata. Quinones naturally occur in many fungi and plants, but among the animals they occur primarily in arthropods and echinoderms (Thomson, 1971). Among animals, only echinoderms are known to have naphthoquinones, but they occur throughout the plant kingdom (Thomson, 1971). More than 200

pigments and metabolites are known to occur in echinoderms, including echinochromes, spinochromes, and gymnochromes (Stonik & Elyakov, 1988; O'Malley et al., 2013).

MacMunn (1885) first reported the presence of myohaematin and histohaematin in the ovaries and stomach-wall of the echinoid *Uraster rubens*. In 1889, MacMunn gave these organic pigments the name of echinochromes, which he claimed gave the sea urchins their red color and acted as oxygen carriers. In 1926, Cannan extracted echinochromes from the eggs, perivisceral fluid, and the test of *Arbacia punctulata* and concluded that if echinochromes do play any role in respiration it is that of an "activator" rather than a carrier of oxygen. Becher et al. (1966) made a note of the inadequacy of the technology used in previous studies to accurately identify these molecules and consequently utilized mass spectrometry to deal with this issue. Becher identified multiple organic molecules, such as naphthazarin from *Echinothrix diadema* spines. These molecules were given the name spinochromes. Further analysis of echinochromes and spinochromes suggested that echinoids and ophiuroids are more closely related to each other and asteroids and holothurians are more related to one another (Singh et al., 1967). This conclusion was based on the presence of closely related, partially methylated derivatives of spinochrome E in the asteroid *Acanthaster planci* and the holothurian *Polycheria rufescens*. Examination of two species of ophiuroids (*Ophiocoma erinaceus* and *O. insularia*) bear a striking resemblance to the pigments in the echinoid *Echinothrix* identified by Moore et al. (1966).

Small amounts of the spinochrome A, a naphthoquinone derivative, were isolated from the arms and pinnules of the crinoid *Antedon bifida* (Dimelow, 1958; Singh et al., 1967). Crinoids are unique in relation to other echinoderms because they produce anthraquinones (Singh et al., 1967). Sutherland isolated a series of anthraquinones from *Comatula pectinata* and

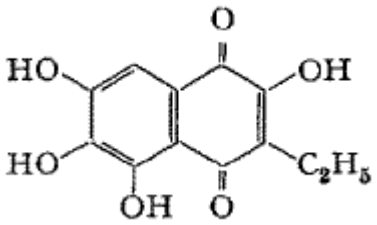
later identified three red anthraquinones (rhodoptilopterin, isorhodoptilopterin, and ptilometric acid) in *Ptilometra australis* (Sutherland & Wells, 1959; Powell & Sutherland, 1967; Figure 3). Ptilometric acid is a 3-hydroxy-propyl anthraquinone equipped with a 2-carboxylic radical that also occurs in both yellow and deep purple specimens of the Australian crinoid, *Tropiometra afra macrodiscus* (Fox, 1976; Takahashi et al., 2002). These anthraquiones are partial methyl ethers of rhodocomatulin (Figure 4). Four groups of polyketide-derived pigments known to occur in crinoids are linear and angular naphtopyrones; 4-acylanthraquinones; 3-alkylanthraquinones; and dimeric bianthrones (Rideout & Sutherland, 1985). De Riccardis et al. (1991) described five violet pigments (gymnochrome A-D and isocymnochrome D) from *Gymnocrinus richeri*. These pigments are a group of brominated phenanthroperylenequinones. Kemami Wangun et al. (2010) identified two additional phenathroperylenequinones from the deep-water crinoid *Holopus rangii*, which have been termed gymnochrome E and F. *Gymnocrinus richeri* serves as an ideal modern analogue to ancient crinoids because it has retained the stalked morphology, unlike modern comatulid crinoids, which is why De Riccardis described it as a so-called “living fossil”.



Ptilometric Acid

Figure 3: Chemical structure of ptilometric acid. The molecular formula is $C_{18}H_{14}O_7$. (Image Source: ChemSpider)

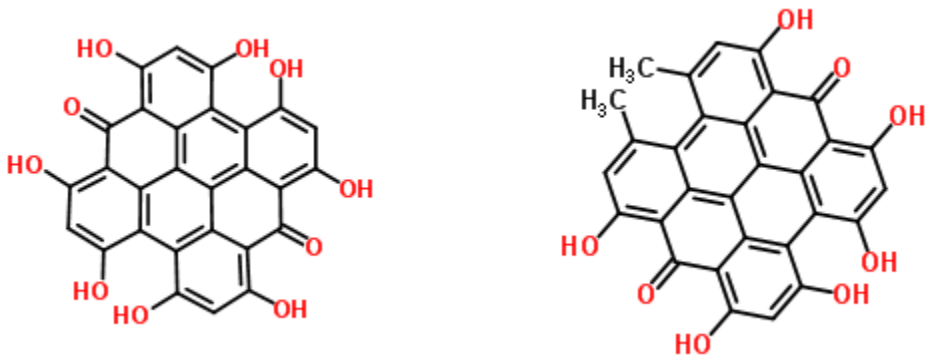
MW: 342. 0739 Da



Rhodocomatulin

Figure 4: Chemical structure of rhodocomatulin (Singh et al., 1966).

Max Blumer pioneered the study of organic molecules preserved in fossil crinoids through his studies of the Jurassic crinoid *Liliocrinus* (formerly *Millericrinus*) from the European Alps (Blumer, 1951). These *Liliocrinus* fossils stood out because of their distinctive violet color. The coloration of these fossils was attributed to organic molecules, which Blumer named fringelites after the village of Fringeli (Blumer, 1951; Blumer, 1960; Blumer, 1962; Thomas & Blumer, 1964; Blumer, 1965; and Hess, 1976). Falk found that fringelite D and hypericin have identical diffuse reflectance UV-Vis spectra, which is not too surprising considering how structurally similar they are with one another (Falk et al., 1994; Falk & Mayr, 1997; Figure 5). Hypericin is a chemical compound extracted from the plant *Hypericum perforatum* (St. John's wort) and is commonly used for its antibiotic properties. Hydroxyphenanthroperylene quinones are known to form extremely insoluble salts with bivalent ions, such as Ca^{2+}, due to the strong acidity of their *bay* region hydroxl groups (Falk & Mayr, 1997; Wolkenstein et al.; 2006). Further, chelation of the *peri*-hydroxyl and carbonyl groups with transition metal ions may lead to further stabilization of the pigments, which justifies their stability through geologic time (Falk & Mayr, 1997; Wolkenstein et al.; 2006).



Fringelite D **Hypericin**

Figure 5: Chemical structures of fringelite D and hypericin. Fringelite D has a molecular formula of $C_{28}H_{12}O_{10}$, whereas hypericin has a molecular formula of $C_{30}H_{16}O_8$. (Image Source: ChemSpider)

Wolkenstein et al. (2006) took a closer look into the relationships between hypericin and the related polycyclic quinone pigments in the Middle Triassic crinoid *Carnallicrinus carnalli* (formerly *Chelocrinus carnalli*) and in the Upper Jurassic crinoid *Liliocrinus*. By use of electron ionization (EI) mass spectrometry and electrospray ionization (ESI) mass spectrometry in association with high-pressure liquid chromatography (HPLC), Wolkenstein et al. (2006) was able to identify a diagenetic pathway for the degradation of hypericin-type compounds in these fossil crinoids. This degradation process has been postulated to be a demethylation sequence associated with a thermal cracking process. A similar thermal cracking process was recognized to occur in the methyl aromatics of petroleum (Behar et al., 1999).

O'Malley et al. (2013) published the most recent analyses on biomarker preservation in Mississippian crinoid fossils from Le Grand, Iowa and crinoids from the Borden Group of Indiana. O'Malley et al. (2005; 2006; 2009; 2013) employed multiple techniques for identifying

organics including Munsel color chart analysis, UV-Vis spectroscopy, fluorescence spectroscopy, and mass spectrometry. Discriminant function analysis of hue, chroma, and saturation gathered from a Munsel color chart sorted the crinoids into subclass level groups (O'Malley et al., 2009). UV-Vis analyses also identified evidence for subclass-level identification. More precise phylogenetic identifications could not be made by mass spectrometry because specific organic molecules were not identified. In fact, O'Malley et al. (2009) recognized the presence of fringelites in the Jurassic crinoid *Liliocrinus*, but fringelites were absent in the Mississippian crinoids analyzed. This raises a question: are the fringelites in Jurassic age crinoids a diagenetic by-product or do they truly reflect biosynthesized organic molecules? Perhaps, fringelites are a more advanced quinone-derived pigment that simply was not biosynthesized by the earlier predecessors to the crinoids of the subclass Articulata. If this were the case it would be possible that the cladids should reflect a more similar array of organic pigments in comparison to those of the subclass Articulata, than crinoids of the subclass Camerata.

Methods

Biomarker Extraction and Isolation

Blumer established a simple extraction method in order to identify the fringelites in Jurassic crinoids (Blumer, 1951). O'Malley et al. (2006) modified this method to analyze organic molecules in Mississippian crinoids and had positive results.

The method that O'Malley et al. (2006) outlined began by first grinding the crinoid samples into a fine powder in order to maximize surface area and hasten the dissolution. 12 N

hydrochloric acid (HCl) was added dropwise to the ground sample until effervescence ceased. This produced an organic residue, which is centrifuged to create a loose pellet of sediment. The solution is then decanted and was described as yellow to orange in color.

In order to separate the organic components from the pelletized residue a series of organic solvents were then added, ranging from very non-polar to increasingly polar. Chloroform was added dropwise to the residue and then allowed to sit overnight. The following day, the solution was centrifuged, and the organic-rich solvent layer was decanted off. This process was repeated with 4:1 acetone: methanol and neat methanol in place of chloroform. Blumer (1951) identified the dominant organic molecules from the fossil material in the 4:1 acetone:methanol solution.

Wolkenstein et al. (2006) employed a different extraction method in order to isolate organics from *Liliocrinus munsterianus* and *Carnallicrinus carnalli*. In this method the sample is first cleaned extensively with acetone in order to remove any contaminants. The sample is then dissolved with 10M HCl. The residues were then centrifuged and rinsed with distilled water. The residue is then dried out overnight at room temperature under vacuum (10Torr). Similar to Blumer (1951) and O'Malley et al. (20013), Wolkenstein et al. (2006) added a series of solvents in order to sequentially extract the organic fractions. Wolkenstein's approach differs from the method utilized by O'Malley et al. (2013) by performing sonication (1h, 40 degrees C) to extract the organics and by using a different series of solvents. The samples are centrifuged in toluene (3x), methanol (3x), and dimethyl sulphoxide (1x).

Simple Extraction

I attempted to modify the previous extraction methods in order to reduce exposure to potentially harmful solvents but had little success in identifying organic molecules in doing so. I initially sought to analyze three species: *Strimplecrinus inornatus, Cribanocrinus watersianus,* and *Elegantocrinus symmetricus*. I began in the same manner as O'Malley (2006) by grinding the fossil material in order to expedite dissolution. I then dissolved approximately 0.5g of each species in a 100mL of 1M HCl overnight. The samples were centrifuged for 20 minutes and the top layer was decanted. I pH adjusted the decanted layer to a basic pH between 8 and 9 and stored the solution in a refrigerator.

Organic molecules were then separated from the residual material with approximately 10mL of toluene. The toluene and residual solid were mixed thoroughly with the assistance of a Vortex and then left to settle overnight. I decanted off the toluene layer and used the UV-vis to search for the presence of organic molecules, but the results indicated a significant inorganic peak and no organic molecules. Over time iron-oxide began to precipitate in the solution.

Soxhlet Extraction

O'Malley et al. (2009; personal communication) suggested Soxhlet extraction as a more efficient and preferred method for the extraction of organic molecules from fossil crinoid samples. The Soxhlet extraction method consists of a heat source, a solvent distillation flask, a glass fiber thimble within a glass apparatus, and a condenser (Figure 6). This method cycles the solvent through a continuous liquid-solid extraction, which recycles the solvent. I used approximately 150 - 250mL of 4:1 acetone:methanol solvent solution as suggested by Blumer and O'Malley. The most important thing to keep in mind in regards to the volume of solvent is

that you need enough solvent in order to continue cycling, although minimizing the amount used aids in hastening the evaporation process. Approximately 0.1g of finely ground fossil material is placed within the glass fiber thimble and was dissolved dropwise with 12M HCl, until effervescence ceases, prior to extraction (Table 3). The amount of HCl used was always less than 1mL. The solvent is then percolated through the sample continuously. O'Malley (personal communication) suggested cycling for a minimum of three hours but allowed her samples to continuously cycle for up to twelve hours. To ensure that I captured a significant amount of organic molecules, while still working in a timely manner I allowed my extractions to cycle for a minimum of seven hours.

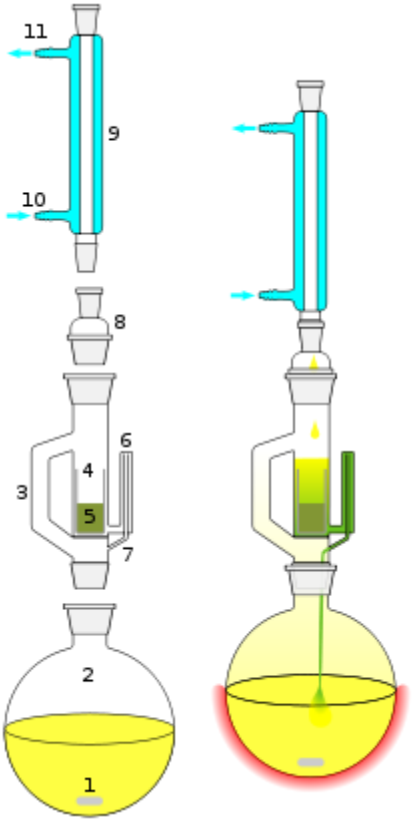


Figure 6: Soxhlet Extractor (Image Source: Wikipedia)

1) Stir bar
2) Round-bottomed flask
3) Distillation path
4) Glass fiber thimble
5) Solid material
6) Siphon
7) Siphon exit
8) Expansion adapter
9) Condenser
10) Cooling water inlet
11) Cooling water outlet

Table 3: Data regarding organic molecule extraction and UV-Vis analyses.

Taxa	Mass of Fossil Material Used for Soxhlet	Part of Crinoid Used	Mass of Solid Extract	Mass of Sample Used for UV-Vis
E. hemisphaericus	0.1208g	Calyx plates	0.1140g	0.0169g
E. symmetricus	0.1024g	Stem	0.1508g	0.0170g
S. inornatus	0.1190g	Stem, calyx, & arms (undifferentiated)	0.1069g	0.0162g
B. rhombiferous	0.1035g	Calyx plates	0.1656g	0.0159g
C. iowensis	0.1064g	Calyx plates	0.1543g	0.0173g
G. tuberosus	0.1084g	Stem	0.1046g	0.0173g

After sufficient cycling the organic-rich solvent is concentrated by use of a rotary evaporation under vacuum. Once concentrated the solution was completely dried in a desiccator and resulted in a solid product, which I used for further analyses. During all extraction methods I made an effort to minimize light exposure because quinones are known to be photolabile (Thomson, 1971).

Biomarker Identification

The identification of these organic pigments in previous studies had been limited by the analytical technology available. In fact, it was predicted that many of the pigments identified are identical (Thomson, 1957; Becher et al., 1966). Bather (1893) made an early attempt to extract organic molecules from *Apiocrinus* in order to account for the purple coloration of the fossils,

but was unsuccessful in his endeavor. It was not until Blumer (1951) analyzed the Jurassic crinoid *Liliocrinus* that organic pigments were isolated by use of UV-Vis and chromatography. As a response to the inadequacy of previous technologies, Becher et al., (1966) employed mass spectrometry as a tool for structural elucidation of the organic molecules in modern echinoderms.

Mass spectrometry has progressed significantly since its early use. There are multiple techniques associated with different phases (gas, liquid, or solid) of the sample. Wolkenstein et al.(2006) compared electron ionization (EI) mass spectra and electrospray ionization (ESI) mass spectrometry for the purpose of analyzing organic pigments in *Carnallicrinus* and *Liliocrinus*. In 2009, O'Malley et al. used electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) to further elucidate upon the structure of the organic molecules.

Other means of identifying organics within echinoderms include fluorescence spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, HPLC, fast atom bombardment mass spectrometry (FABMS), and infrared (IR) spectroscopy. The obvious goal of these methods is to establish what organic molecules are present. This is done by separating the complex solution of organics, generally by use of HPLC, and then reconstructing the structure of the molecule(s) by use of their mass spectra.

UV-Vis

Ultraviolet-Visibility light spectroscopy (UV-Vis) was used to measure light absorbance of the extracted biomarkers. This method has been utilized in order to identify the presence of fringelites (Blumer, 1965; Wolkenstein et al., 2006). UV-Vis serves as a means of rapidly screening for chromophoric organic molecules. In this study a dual-beam Varian Cary 13 UV-Vis spectrophotometer was used with acetonitrile as the matrix. The solutions consisted of 0.01g

to 0.02g of solid extract in an approximately 4mL cuvette (Table 3). All scans were blanked with acetonitrile prior to analyzing the organics. Acetonitrile was used because quinones are poorly soluble in water, yet they are soluble in solvents. The samples were agitated, by stirring and/or shaking, in order to release the organics from the solid extract. Cuvettes were rinsed with methanol (3x) and MQ water (3x) between scans.

Mass Spectroscopy

Orbitrap ESI-MS and ESI-TOF-MS are both suitable for full-spectrum analyses, but there are differences in how this information is acquired. ESI-TOF-MS records how long a particle takes to reach a detector at a known distance. The mass-to-charge ratios of the organic molecules are then determined by the simple concept that heavier molecules travel at slower speeds than lighter molecules. Orbitrap ESI-MS uses an electrode to form an electrostatic field. The ions become trapped because their electrostatic attraction to the inner electrode is balanced by centrifugal forces. The ions then move with different rotational frequencies but with the same axial frequency, which causes ions of different mass-to-charge ratios to spread into rings. Axial oscillations are then detected by their image current, which is subsequently converted to mass spectra via Fourier transform. The differences associated with these methods make comparison between the two unusable, which means that the mass spectra from this study cannot realistically be compared to the mass spectra documented by O'Malley et al., (2013).

In order to determine the mass peaks of the organic molecules present in these fossils Orbitrap electrospray ionization mass spectroscopy (ESI-MS) was employed. The Orbitrap was run in alternating mode in order to capture both the positive ion and negative ion scans. Samples

were introduced by direct injection at a flow rate of 5µm/mL. It is impossible to know the exact concentration of the organics from the solid product, so a solution was made of ~3-4mg of extract in 5mL of acetonitrile. Dissolution was assisted by a Vortex.

Analysis of the first sample, *Strimplecrinus inornatus*, demonstrated that the sample was fairly concentrated. To adjust for this, the next three samples (*Elegantocrinus hemisphaericus*, *Cyathocrinites iowensis*, and *Barycrinus rhombiferous*) were diluted by half with methanol. The orbitrap was cleaned between samples by forced injection of methanol. All scans were conducted at the Campus Chemistry Instrumentation Center (CCIC).

Results

Evaporation of the solvent and biomarker solution yielded a yellow solid product. The UV-Vis scans of all the samples had peaks at similar wavelengths, but the intensities varied between samples. UV-Vis scans indicated that samples that produced a brighter yellow color had more intense peaks, which suggests a greater concentration of organic molecules. *Gilbertsocrinus tuberosus* had peaks at 240nm, 312nm, and 360nm; as well as a shoulder peak at 268nm (Figure 7). *Cyathocrinites iowensis* had peaks at 240nm, 312nm, and 360nm; as well as a shoulder peak at 267nm (Figure 8). *Barycrinus rhombiferous* had peaks at 239nm, 312nm, and 361nm; as well as a shoulder peak at 268nm (Figure 9). *Strimplecrinus inornatus* had peaks at 238nm, 310nm, and 359nm; as well as a shoulder peak at 268nm (Figure 10). *Elegantocrinus hemisphaericus* had peaks at 202nm, 238nm, 310nm, and 360nm; as well as a shoulder peak at

269nm (Figure 11). *Elegantocrinus symmetricus* had peaks at 239nm, 311nm, and 358nm; as well as a shoulder peak at 268nm (Figure 12).

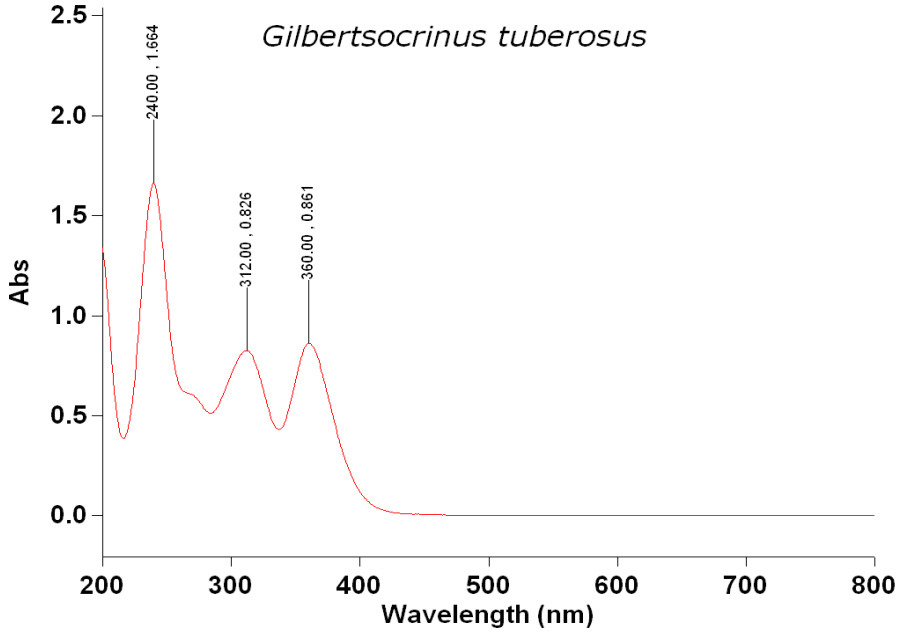


Figure 7: UV-Vis absorbance spectra for *Gilbertsocrinus tuberosus.*

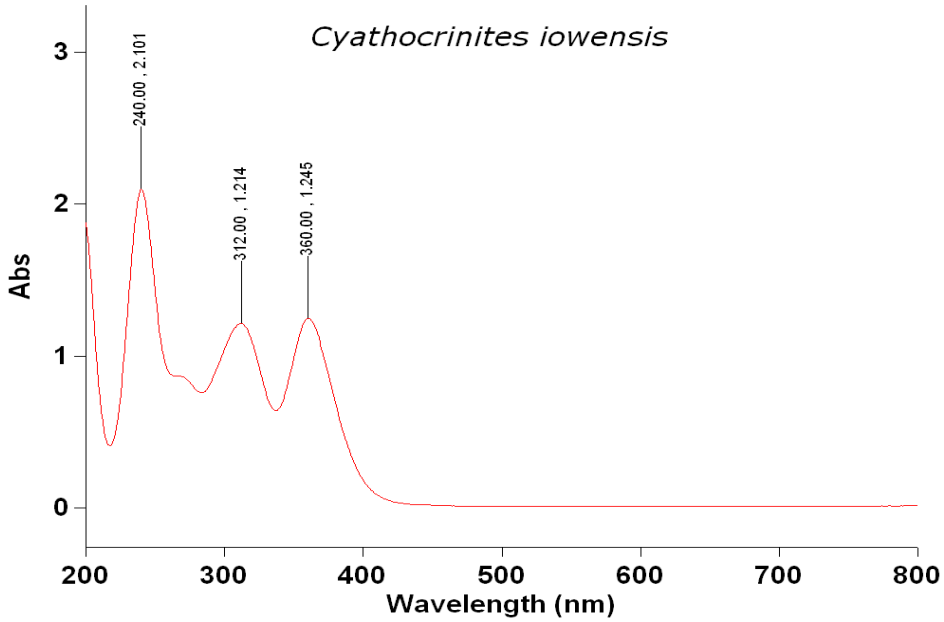


Figure 8: UV-Vis absorbance spectra for *Cyathocrinites iowensis.*

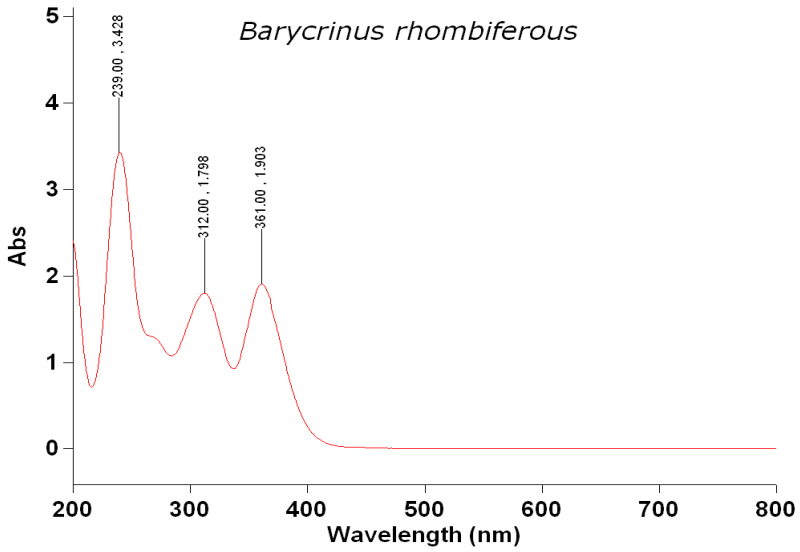


Figure 9: UV-Vis absorbance spectra for *Barycrinus rhombiferous*.

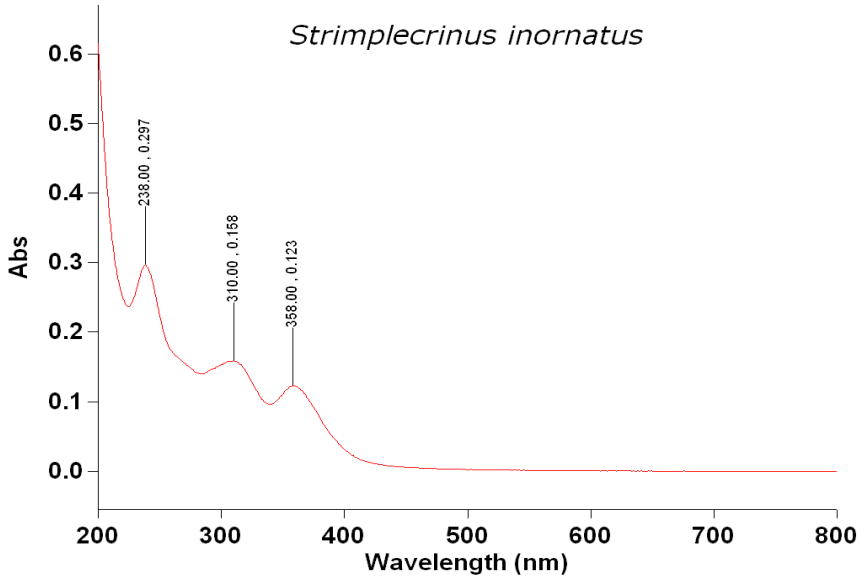


Figure 10: UV-Vis absorbance spectra for *Strimplecrinus inornatus*.

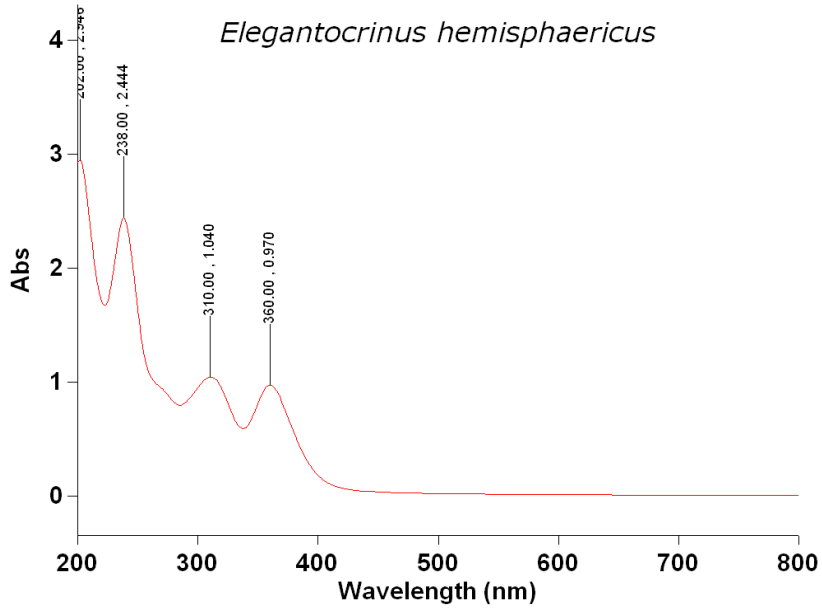


Figure 11: UV-Vis absorbance spectra for *Elegantocrinus hemisphaericus*.

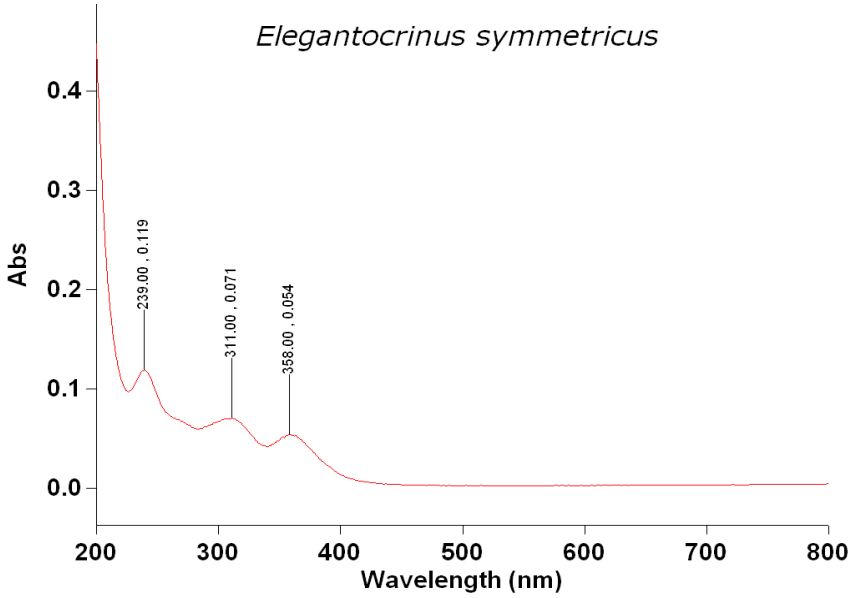


Figure 12: UV-Vis absorbance spectra for *Elegantocrinus symmetricus*.

All of the crinoids in this study seem to have three dominant peaks and one shoulder peak, with the exception of *E. hemisphaericus*, which has an additional peak (Table 4). The dominant peaks can be grouped into the following: peak 1 (238/239/240); peak 2 (310/311/312); and peak 3 (358/359/360/361). The shoulder peaks at 267/268/269 can also be grouped in this same manner. *Elegantocrinus hemisphaericus* is the only species with a unique peak at 202nm, although all graphs do indicate an increase in absorbance around this wavelength. This increase around 200-210nm probably suggests a lower wavelength peak associated with other chromophores, although I am uncertain as to why a peak occurs in *Elegantocrinus hemisphaericus*. Dilution of samples with the highest intensity peaks (*Elegantocrinus hemisphaericus* and *Barycrinus rhombiferous*) indicates a slight shift in the dominant peaks. For this reason, it is justifiable to group the peaks as I have done. In this study, acetonitrile was used as the matrix for these UV-Vis scans whereas O'Malley et al. (2013) used 4:1 acetone:methanol as the matrix, which unfortunately means that comparison between the two is not possible. However, fringelites, represented by peaks at 441nm, 526nm, and 566nm, did occur in the Mississippian crinoids analyzed, in agreement with O'Malley et al. (2013).

Table 4: UV-Vis maximum peaks and shoulder peaks.

Subclass	**Camerata**				**Cladida**	
Peak wavelength	*E. hemisphaericus*	*E. symmetricus*	*S. inornatus*	*G.tuberosus*	*Cyathocrinites iowensis*	*Barycrinus rhombiferous*
202nm	X	-	-	-	-	-
238nm	X	-	X	-	-	-
239nm	-	X	-	-	-	X
240nm	-	-	-	X	X	-
310nm	X	-	X	-	-	-
311nm	-	X	-	-	-	-
312nm	-	-	-	X	X	X
358nm	-	X	-	-	-	-
359nm	-	-	X	-	-	-
360nm	X	-	-	X	-	-
361nm	-	-	-	-	X	X
Shoulder Peak						
267nm	-	-	-	-	X	-
268nm	-	X	X	X	-	X
269nm	X	-	-	-	-	-

Mass spectrometry was utilized for more accurate identification of the organic molecules. Four species were analyzed: *Elegantocrinus hemisphaericus, Strimplecrinus inornatus, Barycrinus rhombiferous,* and *Cyathocrinites iowensis.* These four taxa offer the opportunity to compare two orders from two different subclasses. All samples were extracted and stored in acetonitrile.

Positive ion mode scans revealed a striking similarity between the two cladid crinoids: *Barycrinus rhombiferous* and *Cyathocrinites iowensis*. These two species share five peaks at mass-to-charge ratios (*m/*z) of 116.1, 188.1, 229.1, 385.5, and 441.5. They also share an additional *m/z* at 550.8, but this is thought to be an artifact because the peak persisted in the methanol blanks. This artifact is also present in the positive ion mode mass spectra of *Elegantocrinus hemisphaericus*. The negative ion mode scans also reveal a similarity between *B. rhombiferous* and *C. iownesis* with a shared dominant *m/z* at 294.6/294.7. Conversely, there did not appear to be many similarities expressed between the camerate crinoids: *E. hemisphaericus* and *Strimplecrinus inornatus.* In fact, the two species did not share any exact *m/z* in either the positive or negative ion mode scans (Figure 13-20). This implies that *E. hemisphaericus* and *S. inornatus* are not as closely related to one another as *B. rhombiferous* and *C. iowensis*, which is not very surprising considering that *E. hemisphaericus* and *S. inornatus* are from different monobathrid crinoid clades.

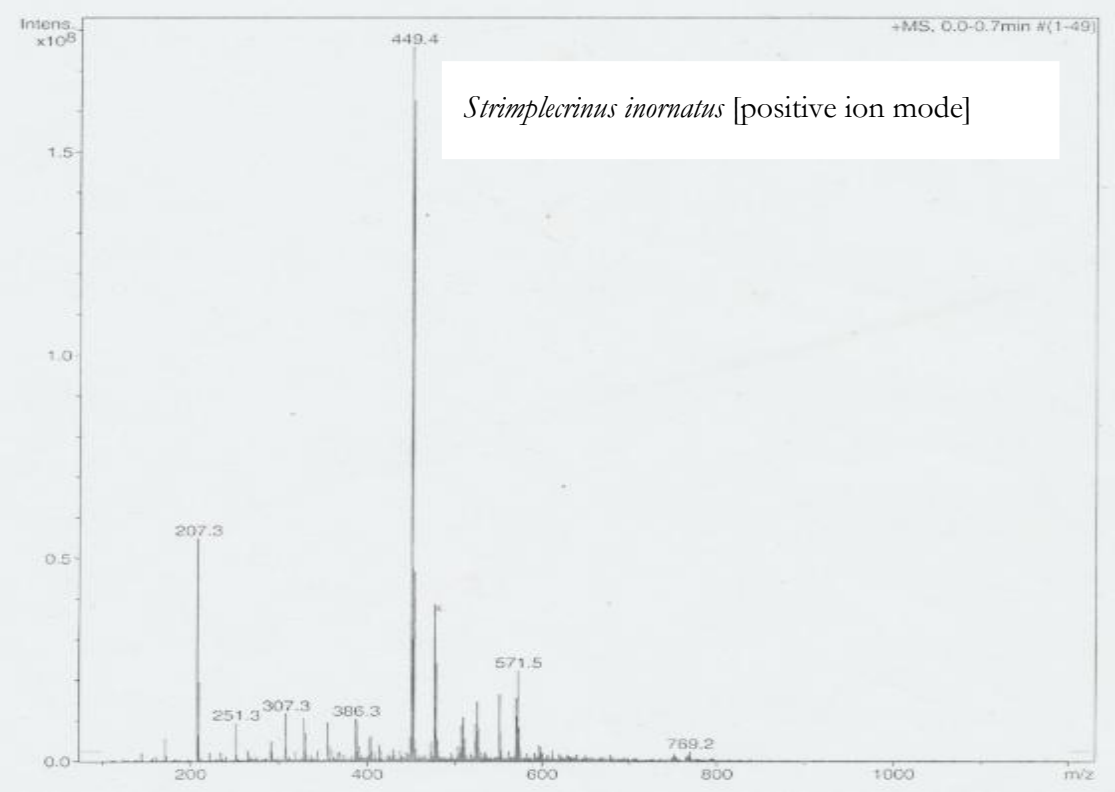


Figure 13

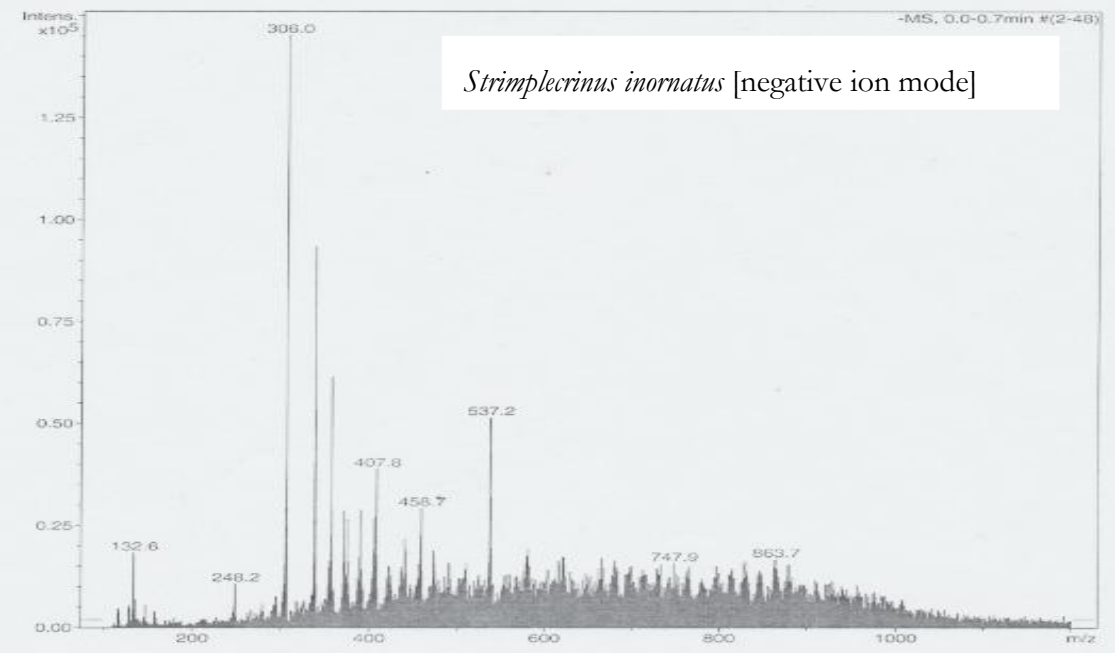


Figure 14

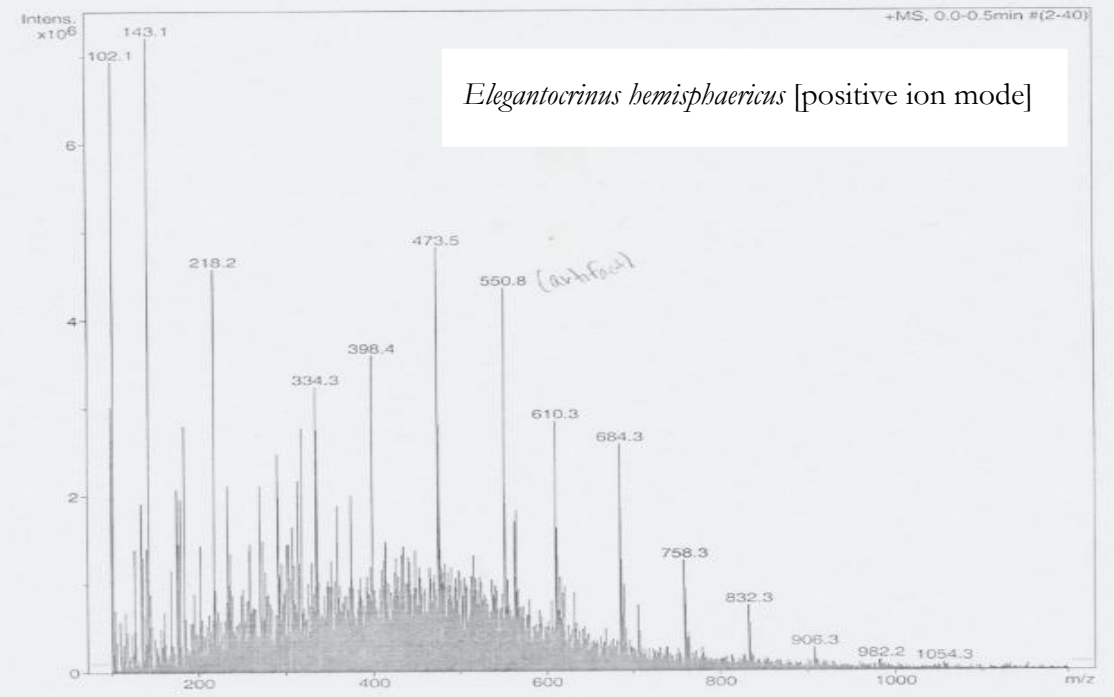


Figure 15

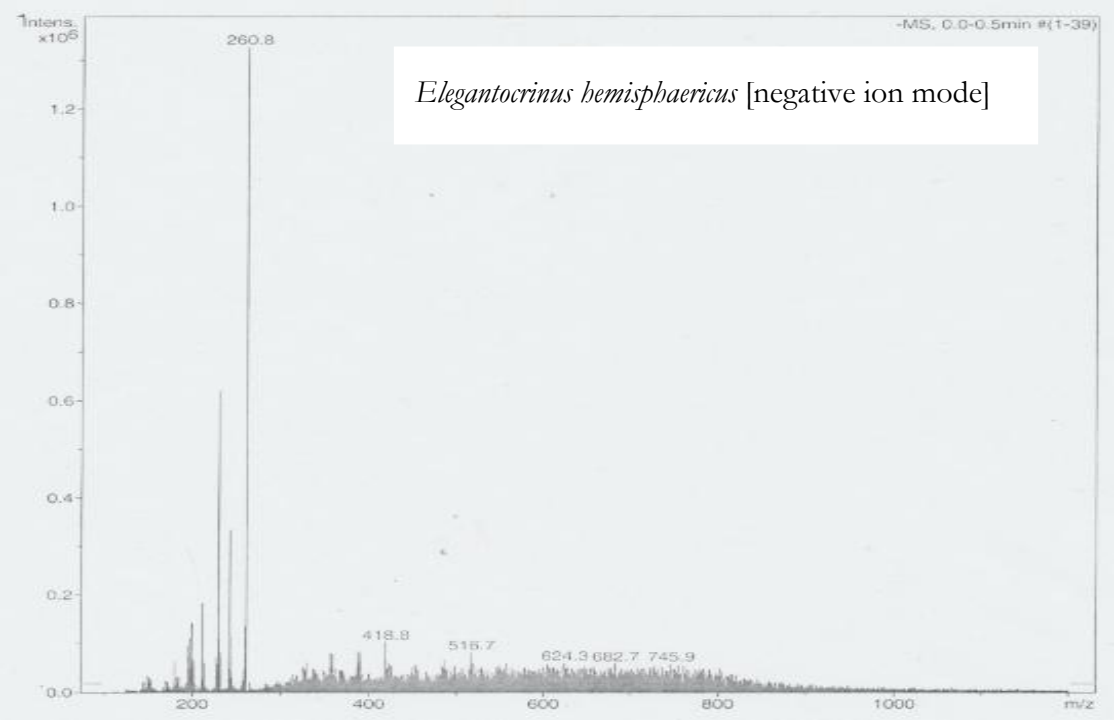


Figure 16

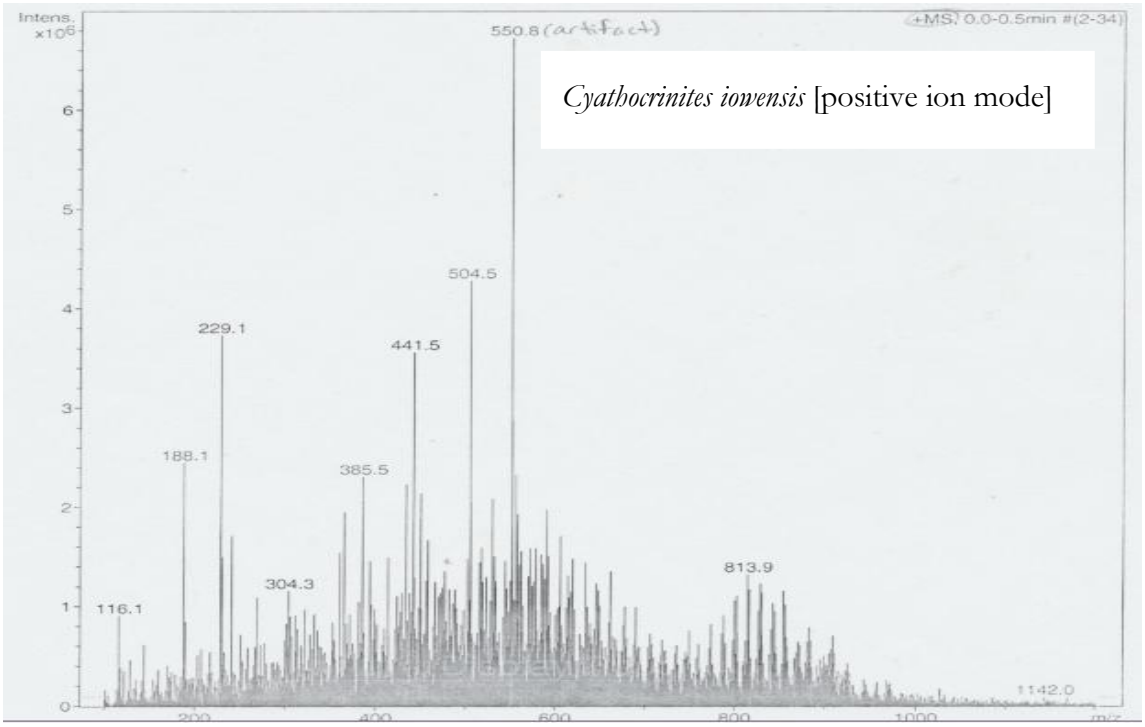


Figure 17

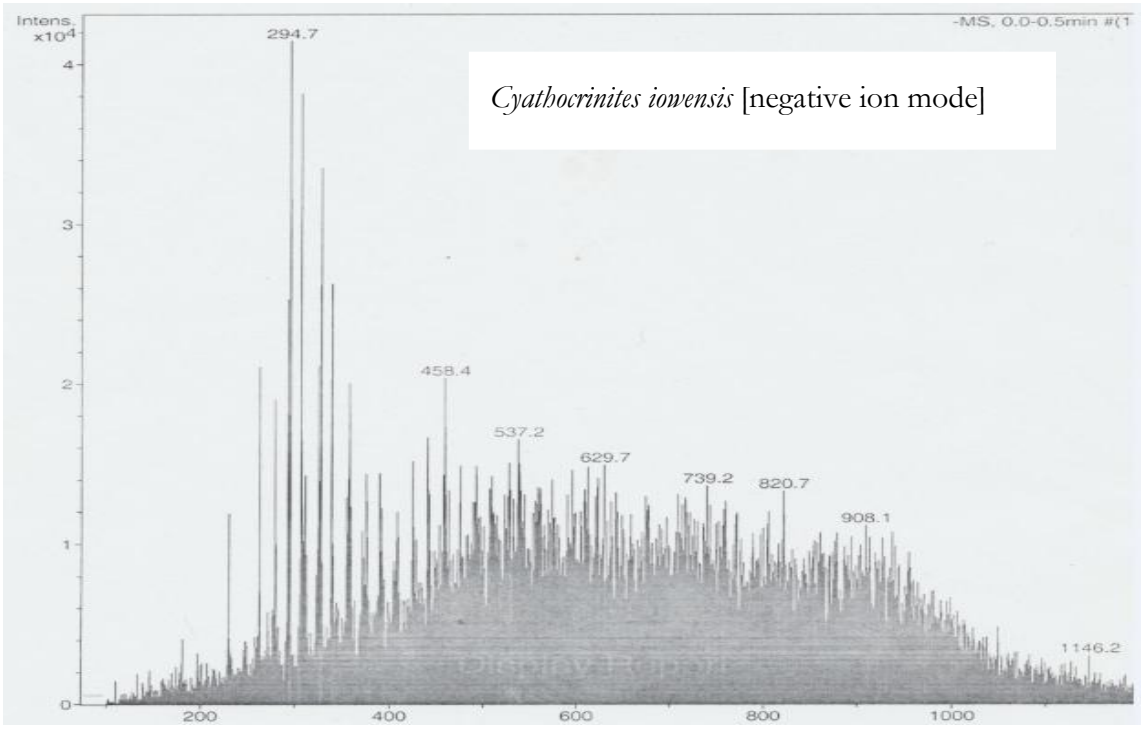


Figure 18

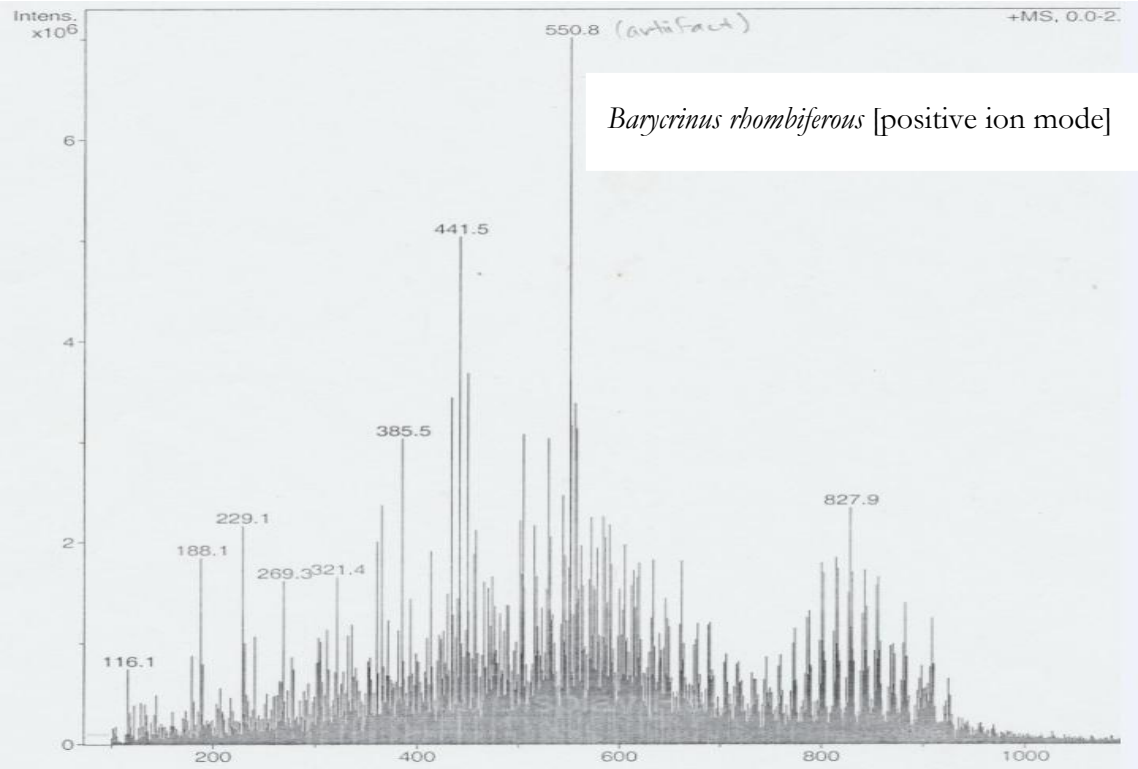


Figure 19

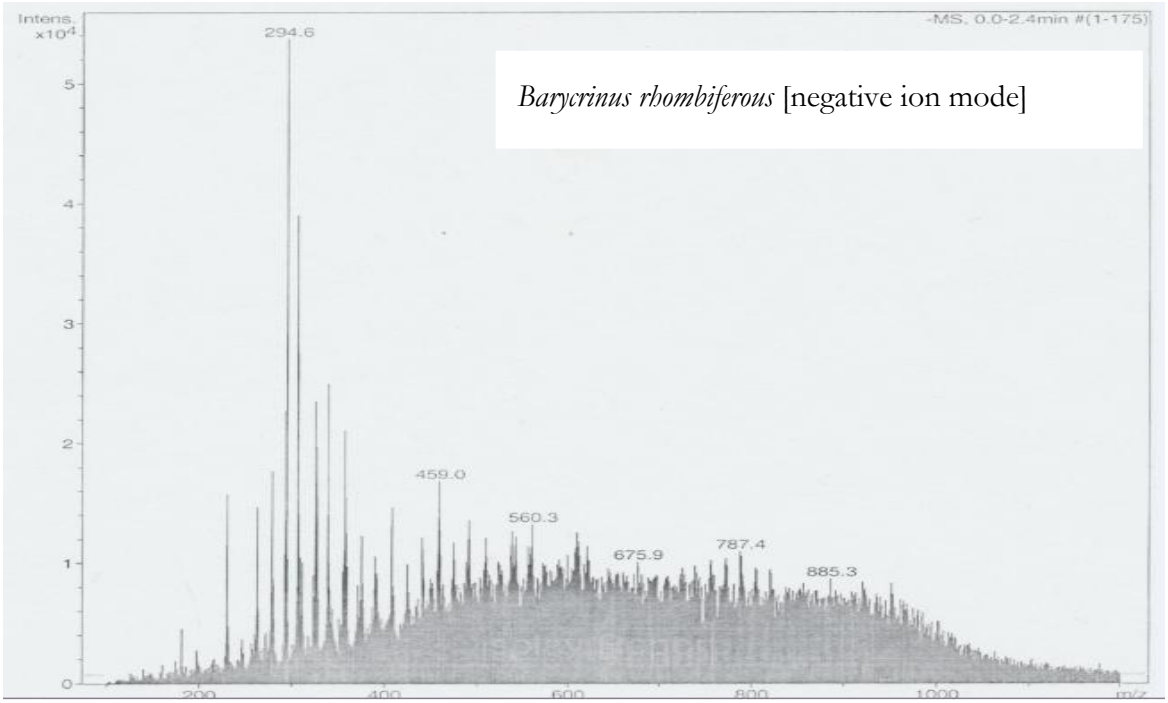
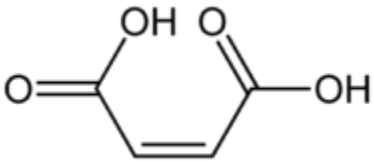


Figure 20

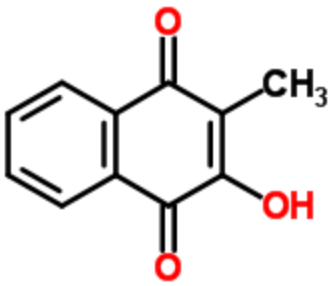
Identification of the molecules present was difficult because the samples consisted of complex solutions that require NMR and/or HPLC analyses. However, predictions can be made based on comparisons to mass peaks. The 116.1 *m/z* shared by *B. rhombiferous* and *C. iowensis* may be maleic acid, which is a dicarboxylic acid similar to the 2-carboxylic radical that occurs in ptilometric acid isolated from *Ptilometra australis* and *Tropiometra afra macrodiscus* (Figure 21). The 229.1 *m/z* shared by *B. rhombiferous* and *C. iowensis* may be similar to 2,4,6-trinitrophenol, also known as picric acid. 2-Hydroxy-3-methyl-1,4-naphthoquinone has a monoisotopic mass of 188.0473Da, which may correspond with the *m/z* at 188.1 shared by *B. rhombiferous* and *C. iowensis* (Figure 22). The *m/z* at 385.5 that occurs in *B. rhombiferous* and *C. iowensis* may be similar to bianthrone, also known as dianthraquinone, which has a molecular weight of 384.43 (Figure 23). Bianthrone has been identified in comatulid crinoids by Rideout & Sutherland (1985). The 504.5 *m/z* that occurs in *C. iowensis* may be associated with hypericin, which has a molecular weight of 504.44Da (Figure 5). Another comparable molecule is 9,10-Anthraquinone which has a monoisotopic mass of 208.0524Da. The anthraquinone may correspond with the 207.3 *m/z* that occurs in *S. inornatus* (Figure 24). The 863.7 *m/z* that occurs in the negative ion mode of *S. inornatus* may be associated with coenzyme Q10, ubiquinone, which has a molecular weight of 863.34Da. However, coenzyme Q10 is extremely labile and thus, is unlikely to be preserved in fossil remains.



Maleic Acid

Figure 21: Maleic acid chemical structure. (Illustration by Ben Mills).

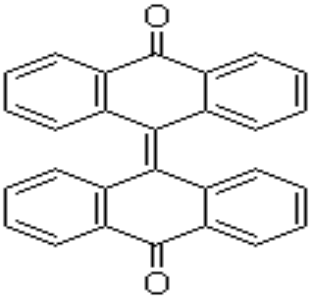
MW: 116.1 Da



2-Hydroxy-3-methyl-1,4-naphthoquinone

Figure 22: Chemical structure of 2-Hydroxy-3-methyl-1,4-naphthoquinone. The molecular formula is $C_{11}H_8O_3$. (Image Source: ChemSpider)

MW: 188.0473 Da



Bianthrone

Figure 23: Chemical structure of bianthrone. The molecular formula is $C_{28}H_{16}O_2$. (Image Source: ChemBlink)

MW: 384.43 Da

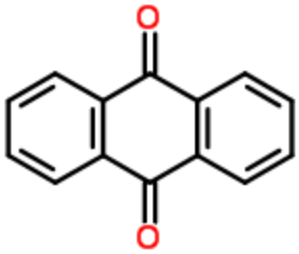


Figure 24: Chemical structure of 9,10-Anthraquinone. The molecular formula is $C_{14}H_8O_2$. (Image Source: ChemSpider)

MW: 208.0524 Da

9,10-Anthraquinone

Discussion & Conclusions

Biomarker Preservation

Three primary factors influence the preservation of biomarkers in these fossil crinoids. The first factor is the geologic setting in which these Mississippian crinoids are deposited. All taxa from this study lived in epicontinental seas. The crinoids were all rapidly buried by fine-grained sediment, which preserved the fossil material by encapsulating it and preventing leaching of fluids. Also, the geologic setting of these fossils has not undergone significant tectonics or metamorphism that could have destroyed the fossil material. Evidence of this is provided by a conodont alteration index of 1.5 or less (O'Malley et al., 2013). In contrast with this, the samples analyzed by Wolkenstein et al. (2006) come from a tectonically active area, which could have altered the organic molecules that are preserved in the fossils. This may be why Mississippian crinoid fossils do not reflect the presence of fringelites.

The second major factor is the stereomic structure of the crinoid test that is infilled by stroma while alive. During early diagenesis, traces of the stroma are preserved in calcite cement

that rapidly occludes the pores of the stereom. The calcite cement forms the "crystal caskets" that were described earlier. These crystal caskets also aid in inhibiting leaching and further diagenesis (Dickson, 2001).

The third, and final, factor impacting the preservation potential of these organic molecules is chelation. Chelate bonds occur with transition metals at the *peri*-hydroxl and carbonyl groups in hypericinoid pigments, which results in the formation of insoluble salts (Falk & Mayr, 1997). These insoluble salts are geochemically stable and inhibit dissolution by leaching (Wolkenstein et al., 2006). Despite the fact that these three factors aid in preserving these organic molecules, it is still reasonable to assume that some diagenetic alteration will occur over a geologic time span.

Phylogeny

Although organic molecules can be extracted from all parts of the crinoid (root, stem, and crown), the distribution of organic molecules within a single crinoid may be variable. The modern stalked crinoid *Gymnocrinus richeri* was noted to have a saffron yellow "body" (calyx), a darker yellow stalk, and dark yellow-green "tentacles" (arms) while living (De Riccardis et al., 1991). Wolkenstein et al. (2006) also noted that the intensity of coloration varied in different parts of the crinoid. The holdfast had the most color, which is likely due to constant burial in the substrate. For this reason, comparisons of pigments from the stem and calyx may not be practical. In spite of this, the samples used in this study depicted consistent coloration throughout the specimen, which indicates a uniform dispersal of organic remains in the calcite stereom. The striking similarities in the UV-Vis absorbance spectra of the Mississippian crinoids suggest that organic molecules from the calyx, arms, and stem are comparable. An analysis comparing the

distribution of organic molecules in different parts of the crinoid is the only way to be certain of this.

Despite the occurrence of organic molecules in all taxa analyzed, the use of preserved organic molecules in fossil crinoids as a means of reconstructing phylogeny requires further study. Rapid screening by UV-Vis did not produce spectra that were phylogenetically significant in all cases. The similarities expressed between the taxa from the subclass Cladida (*Barycrinus rhombiferous* and *Cyathocrinites iowensis*) suggested that mass spectra may offer a plausible approach to reconstruct phylogeny. The mass spectra of the monobathrid crinoids, *Elegantocrinus hemisphaericus* and *Strimplecrinus inornatus*, did not reflect any shared organic molecules; however this may be attributed to the complex solution of organic molecules. Initial separation by liquid chromatography (LC) may reveal related molecules between these species that were masked by the complex solutions. Nonetheless, organic molecules may offer a secondary character in instances where morphological characters are questionable in confirming phylogeny.

A database of species-specific organic molecules isolated from echinoderms would strengthen the potential for phylogenetic reconstructions via organics. PathCase, an online database, has a list of 109 biosynthetic/degradation pathways associated with organic molecules from the purple sea urchin *Strongylocentrotus purpuratus*. As this database grows, perhaps phylogeny of echinoderms by use of organic molecules may become more realistic. The use of organic molecules in class-level phylogeny of echinoderms was implied by Singh et al. (1966) through the notion that holothurians and asteroids are more closely related to one another and echinoids and ophiuroids are more closely related to each other. Therefore, the concept of biomarkers as phylogenetic characters in fossil echinoderms should not be disregarded; rather

this potential should serve as motivation to fill in the gaps of information that impede the application.

Biosynthesis & Function

The occurrence of polyketide sulphate esters has been documented extensively in modern crinoids (Rideout et al., 1979; De Riccardis et al., 1991; Takahashi et al., 2002). It has been recognized that polyketide synthases form in marine invertebrates, such as crinoids, through the acetate-malonate pathway (Thomson, 1971; Shen, 2000). Salaque et al. (1967) suggested that biogenesis may proceed in two stages, first cyclization of the polyketide and then the introduction of the side chain, for the formation of spinochromes.

These molecules have been suggested to function as anti-feedants (Rideout et al., 1979; Falk, 1999; Takahashi et al., 2002). Fringelites are postulated to act as a photosensory system in crinoids because excessive amounts of hypericin ingestion results in hypersensitivity to light in domesticated animals (Falk, 1999; O'Malley et al., 2006). Such a system would only be useful at depths at which light is able to penetrate. Thus, a photosensory system would be more effective in a modern comatulid crinoid. This is supported by the fact that many comatulid crinoids hide during the day and feed at night.

Others have suggested that the organic molecules have a respiratory function in echinoderms (McMunn, 1889; Cannan; 1927). These early attempts at determining the function of the organic molecules have either been disproven or lack enough evidence to be certain of such a claim. Yet, a protein, NADH-ubiquinone oxidoreductase chain 2, was identified in *Florometra serratissima* and was suggested to play a role in respiration (Scoura & Smith, 2001).

Ubiquinones are biosynthesized by a mevalonate pathway and are known to act as electron carriers in aerobic cellular respiration and photosynthesis (Thomson, 1971).

Diagenesis

The conversion of the high-Mg calcite test to low-Mg calcite during early diagenesis has already been discussed, but over such a long period of time further diagenesis should be expected to some extent. It is possible that the organic molecules identified in Mississippian crinoids are fragments of the polyketide sulphate esters documented in modern crinoids. Wolkenstein et al. (2006) proposed a diagenetic pathway from hypericin to hexahydrophenanthroperylene (HHPP) (Figure 25). Wolkenstein et al. (2006) also recognized that the pigment concentrations of the Jurassic *Liliocrinus* were approximately an order of magnitude higher in concentration than the Triassic *Carnallicrinus*, which was attributed to a degradation of organic matter on a geologic time scale. If this is the case it should be anticipated that organic matter from the Mississippian should have undergone further diagenetic change.

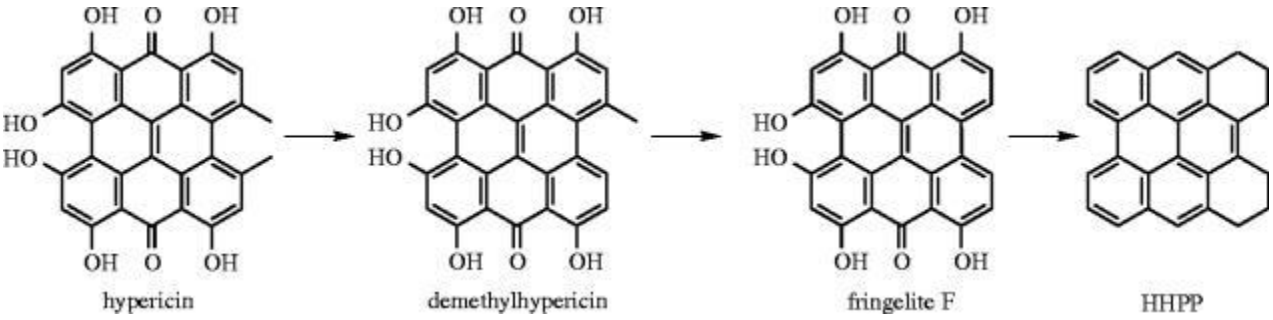


Figure 25: Proposed diagenetic pathway for the degradation of hypericin in Mesozoic crinoids (Wolkenstein et al., 2006).

The degradation pathway in Figure 25 shows the reduction of side chains, which is essentially the reverse sequence described for biosynthesis of these molecules. In fact, Blumer

(1965) suggested that fringelites may be the result of reduction and condensation reactions of anthraquinones. However, the final product of HHPP is aromatic and would not likely reduce further to produce kerogen as suggested by Wolkenstein et al. (2006). It is unlikely that hypericin could degrade to form the molecules that are identified in Mississippian crinoids, which suggests that the Mesozoic crinoids express different polyaromatic hydrocarbons than those in Paleozoic crinoids. This difference in organic molecule assemblages could be associated with a biological difference between Mesozoic and Paleozoic crinoids, but more likely is attributed to differences between the geologic settings that these fossils were deposited in.

Future Work

Through this study I found that Soxhlet extraction is more effective than simple extraction, in accordance with O'Malley et al. (2009, 2013), however the ultrasonication method that Wolkenstein et al. (2006) employed may be a preferred method over Soxhlet extraction. The Soxhlet extraction method takes a significant portion of the day (up to 12 hours) and is followed by evaporation, which is another time consuming process (up to 6 hours). The benefit of this method is that it is low maintenance, which means after the Soxhlet has been setup you are free to conduct other analyses. Despite this, the rapidness of ultrasonication (1 hour) allots for significantly faster extraction, which provides extra time for further analyses. In fact, Marvin et al. (1992) wrote a paper on this very topic and found that sequential ultrasonic extraction with two solvents was much more rapid (45 minutes) than Soxhlet extraction (two days) and resulted in equal extraction efficiency. However, personal communication with Dr. Chin suggests that ultrasonication may alter the organic molecules because it is characteristically destructive. A study analyzing the extraction efficiency of simple extraction, Soxhlet extraction, and

ultrasonication from organic molecules in fossil material could determine the ideal extraction method.

Another future study that could be useful has already been mentioned. That is a study that analyzes the distribution of organic molecules from the holdfast to the pinnules on the arms of a single species. Because others have noted different colors associated with different parts of the crinoid (De Riccardis, 1991; Wolkenstein et al.; 2006), there could very well be variability between different segments of the crinoid. Further elucidation of the organic molecules present should also be included in this study by use of HPLC, fluorescence spectroscopy, and NMR. In March, Dr. Ausich and students from his lab group collected samples of modern echinoderms in San Salvador in order to analyze Mg/Ca ratios. The acquisition of these samples offers an opportunity to analyze the distribution of organic molecules in modern and ancient echinoderms.

There are numerous questions still unanswered about these organic molecules and their stability over geologic time. There are two approaches that I would suggest in order to further elucidate the diagenetic pathway of these organic molecules. First of all, I suggest analyzing a single species that displays a variety of preservation quality. Analyses of a spectrum of preservation quality from pristine to poor could provide insight into the range of diagenetic products. For this approach, the goal would not be to find the taxa representing the most vibrant colors, but rather those that depict the greatest range of coloration. The second approach that I suggest is to broaden the spectrum of taxa analyzed through geologic time. Currently, there have been studies that analyzed organic molecules in echinoderms from the Holocene, Jurassic, Triassic, and the Mississippian. Filling in the gaps and even looking further into the geologic past may provide the necessary information in order to establish a diagenetic pathway on a geologic time scale. Personal communication with Mark Peters suggested that organic molecules

may be preserved in the Ordovician crinoid *Merocrinus*. Specimens of this genus occur in the Cincinnatian of Ohio displaying a red color that stands out from the blue-gray shale that they are deposited in; however this coloration may simply be iron oxide.

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